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Synthesis of methods used to assess soil protease activity

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ABSTRACT

Proteases play a crucial role in the soil nitrogen (N) cycle by converting protein to oligopeptides and amino acids. They are often viewed as a bottleneck in terrestrial N cycling; therefore, it is vital that we have robust methods for evaluating protease activity in soil to understand global patterns of protease activity. In response to this, several laboratory-based protease methods have been developed and subsequently modified. However, the validity of these different approaches remains largely unknown. In addition, the lack of standardised protocols makes it difficult to compare protease activity across studies. In this systematic synthesis, we critically evaluate the most common colorimetric and fluorimetric methods used to measure soil protease activity involving 680 independent studies and 1,491 individual assays. To investigate the key regulators of soil protease activity, we collected associated metadata on environmental (mean annual temperature and soil pH) and methodological (assay temperature and pH) factors. Protease activity measured with colorimetric substrates were centred around ca. 1000 nmol product g^{-1} h^{-1}, whilst rates measured with fluorimetric substrates were lower at ca. 100 nmol product g^{-1} h^{-1}. Fluorimetric and colorimetric substrates target different proteases which are likely to have different abundances, kinetic parameters, catalytic mechanism or ecological function suggesting why colorimetric substrates have a higher protease activity. We found soil protease activity varied widely around these peaks, likely due to a wide range of environmental or methodological factors that may influence/bias the result. We present the following recommendations for measuring soil protease activity: 1) report assay conditions and soil characteristics, particularly pH and temperature, 2) conduct the assay at either field or optimised pH and temperature conditions, and, 3) check that measurements lie between 0 and 5000 nmol product g^{-1} h^{-1}. This will help reduce the variation in soil protease activity measurements due to methodological bias and improve reporting of abiotic and biotic associated data. Altogether this will lead to a better understanding of the ecological drivers of protease activity and refine parameterisation of global biogeochemical models.

1. Introduction

Protease activity is an important process in the soil N cycle and is often considered to be the rate-limiting step of N mineralisation (Jan et al., 2009; Weintraub and Schimel, 2005). Proteases catalyse the hydrolysis of proteins and polypeptides into oligopeptides and amino acids. In the soil, extracellular proteases are released by microorganisms, plants, animal excrements and leached from agro-industrial fertilisers, though microorganisms are considered the dominant producer (Greenfield et al., 2020a; Vranová et al., 2013). Protease activity, alongside other enzymes, are increasingly being used as soil quality indicators to evaluate how well a soil is functioning i.e. more microbial activity indicates a well-functioning soil (Schloter et al., 2018; Trasar-Cepeda et al., 2008). Therefore, standardised soil sample pre-treatment, assay protocol and measurement units are vital to ensure comparability across studies.

Methods used to assay soil protease activity can be split into two main categories: fluorometric and colorimetric analysis. Both methods are based on the addition of a substrate bonded to a fluorophore or chromophore which is added to the soil solution or soil slurry and the breakdown products are then measured directly or indirectly. Fluorometric assays are more sensitive than their colorimetric counterparts with a limit of detection around 50 pmol of substrate (Deng et al., 2013; Dick et al., 2018). However, both are susceptible to interference from other soil components (e.g. humic compounds), which must be accounted for (Deng et al., 2013). Both techniques offer substrates that

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can be measured using 'bench-top' or 'microplate' based protocols with the latter allowing for a larger number of low volume samples to be processed but can incur more measurement error e.g. in pipetting (Bell et al., 2013; Deng et al., 2013). The proteases targeted by an assay method tend to fall into two distinct categories related to the substrate used: 1) specific proteases, and 2) non-specific proteases. For example, the leucine-7-amino-4-methlycoumarin (AMC) substrate is hydrolysed by leucine aminopeptidase (EC 3.4.11.1) which preferentially catalyses the hydrolysis of leucine at the N-terminus of polypeptides and proteins. It is worth noting here most fluorimetric enzyme assays use the fluorescent compound 4-methlumbelliferone (MUB), however, protease assays use 7-amino-4-methlycoumarin (commonly referred to as AMC or MUC). In contrast to AMC, the substrate casein, is cleaved in several sites by casein-hydrolysing peptidases like trypsin (EC 3.4.21.4). These indirect methods of analysis tend to measure non-specific proteases. Despite being one of the oldest techniques (Ladd and Butler, 1972), colorimetric analysis with casein remains a popular method due to its broader analysis of soil proteases. However, little work has been done to determine whether different substrates provide a similar estimate of soil protease activity and organic N processing rates in soil.

Another key difference in protease assay methods is whether the assay is conducted under 'laboratory-optimised conditions' or 'field-relevant conditions'. The former normally involves optimisation of the pH, temperature and substrate conditions to maximise catalytic activity (Tabatabai, 1994). In the field, soil pH varies greatly according to soil type, land use and pollution events while temperature is determined by season, altitude, and climate (Slessarev et al., 2016). The optimum pH of protease activity is around 7 and a temperature around 40–60 °C although this can vary depending on the soil, location, and microbial origin of the protease (Ladd and Butler, 1972; Puissant et al., 2019; Vazquez et al., 2005). Therefore, the choice of field-relevant or laboratory-optimised conditions is likely to have a marked effect on the measured soil protease activity and thus the interpretation of the results obtained.

The wide range of assay methods and substrates used leads to questions over how comparable studies are. There have been many objective reviews over the years that conclude the need for standardisation of methods when measuring soil enzyme activity (e.g. Dick, 2011; Fornasier et al., 2011; German et al., 2011; Nannipieri et al., 2017). Despite these reviews, standardisation and transparency of reporting key methodological and environment variables that affect enzyme activity is lacking. Furthermore, there are no commercially available reference materials for quality assurance purposes (e.g. standard soil proteins or soil proteases) and no standard reference values for protease activity for use as a soil health metric. This has led to the publication of questionable results, exacerbated by pitfalls in methodologies and activity calculations (German et al., 2011).

The aim of this study was to 1) review current colorimetric and fluorimetric methods used to measure protease activity in soil, 2) report the use of field-relevant versus laboratory-optimised pH and temperature conditions in protease assays, 3) evaluate the numerical range of soil protease activity at a global scale, 4) identify potential factors that may help explain the natural variation in protease activity, and 5) provide guidance for future measurements and reporting of soil protease activity.

2. Methods

2.1. Systematic review and data exportation

We conducted a systematic review in March 2020 to obtain studies that had measured soil protease activity. We used Web of Science as our primary database and used the search string "TS = (soil* NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase* OR proteolytic OR assay? OR enzyme? OR "enzyme activity"))" (see Table S1 for search term strategy). Following this, we used ScienceDirect to search full texts for common assay substrates ("soil" followed by "7-Amino-4-Methylcoumarin", "N-benzoyl L-arginine amide", "casein", "gelatin", "*p*-nitroaniline", "haemoglobin" and "benzyloxycarbonyl phenylalanyl leucine", "azocol" and "azocasein"). We also searched common synonyms and acronyms for these substrates and filtered results for journals in the relevant discipline (Table S2). Studies were selected using predetermined criteria (Table S3) and in total, 680 studies met the criteria for inclusion (Fig. S1. PRISMA diagram). Once these studies were selected, we exported data into an Excel spreadsheet. We exported data on methodological and environmental factors: substrate used, assay pH, assay temperature, mean soil protease activity and its standard deviation, soil pH, sample location (geographical coordinates) and mean annual temperature (MAT). Raw data along with referencing information can be found in Greenfield et al. (2021). Mean soil protease activity data was exported using predetermined criteria namely soil protease activity from controls on a dry weight basis that could be converted into nmol product g^{-1} h^{-1} (Table S4). Studies that did not meet criteria for soil protease activity data were not used in analysis for aims 3 and 4 of this study. When more than one assay was included in a study (e.g. studies that measured different soil types or under different assay conditions) they were counted as an independent protease activity measurement. Therefore, from the 680 individual studies collected there were 1,491 individual assays. Protease activity was converted into nmol product g^{-1} h^{-1} (where applicable) and studies were grouped according to the substrate used: 7-Amino-4-Methylcoumarin (AMC), casein, N-benzoyl L-arginine amide (BAA), benzyloxycarbonyl phenylalanyl leucine (Z-phe-leu) and *p*-nitroaniline (pNA). We acknowledge that AMC, Z-phe-leu and pNA come in multiple forms, but for the purposes of this study we grouped the variations of each of these substrates (Table S5). We have not analysed or reported on the substrates azocoll, azocasein, haemoglobin, gelatin and native (no substrate) because these were represented by < 10 studies in the dataset.

2.2. Data analysis

All data analysis was conducted in R 3.5.0 (R Core Team, 2018) and all graphs were drawn using the package *ggplot2* (Wickham, 2016). Protease activity values were converted to nmol product g^{-1} h^{-1} in order to compare within substrates (product = AMC, tyrosine, NH_4^+, leucine and pNA for AMC, casein, BAA, Z-phe-leu and pNA substrates). Outliers were removed by estimating the maximum activity possible for the assay based on the amount of substrate added (i.e. theoretically impossible values where the reported protease activity exceeded the amount of substrate added were not deemed scientifically credible and were thus omitted). From this method of outlier removal, we excluded 103 assays from 53 studies from analysis for aims 3 and 4 (i.e. 7.8% of the total studies; Fig. S2). Mean annual temperature (MAT) data was extracted using packages *sp* and *raster* in R according to the GPS coordinates for assays that reported no MAT (Bivand et al., 2013; Fick and Hijmans, 2017; Hijmans, 2020; Pebesma and Bivand, 2005). The pH optima of leucine aminopeptidase measured by Puissant et al. (2019) at pH 7 was used to determine the difference between assay pH reported and pH optima of the enzyme. Although we acknowledge that leucine aminopeptidase does not represent the pH optimum of all protease enzymes targeted by the substrates analysed in this study, there is little information on the soil pH optimum of the other proteases targeted by casein, BAA and Z-phe-leu substrates. Work by Ladd and Butler (1972) suggests the pH optimum is between 6.8 and 8.8 for Z-phe-leu, BAA and casein. Other studies including Niemi and Vepsäläinen (2005) and Sinsabaugh et al. (2008) have measured a pH optimum of approximately 7 for leucine aminopeptidase.

A linear regression model was used to determine the extent to which environmental (soil pH and MAT) and methodological factors (assay pH and assay temperature) explain the variation in protease activity across studies. A linear model using the function *lm* was built to test the effect of environmental and methodological factors on mean soil protease

activity collected from the studies. Protease activity measurement of each assay was only included in linear regression analysis if there was a value for mean soil protease activity, MAT, soil pH, assay pH and assay temperature. The linear regression models were tested for normality using the Shapiro-Wilks test and then visually assessed using a *qqnorm* plot of the residuals. In order to meet normality assumptions of the model, soil protease activity was $\log_{10}$ transformed.

3. Results

3.1. Soil protease activity methods

3.1.1. Fluorimetric-based protease assays

Fluorimetric assays for quantifying soil enzyme activity were first introduced by Pancholy and Lynd (1972) to measure soil lipase activity. However, our analysis shows that their use for measuring soil protease activity did not become commonplace until the 2000s (Fig. 1). Since their introduction, the use of fluorescent substrates has become increasingly popular. Overall, our analysis suggests that they account for 40% of the total soil protease studies, while in the last 5 years they account for nearly 60% of the total. Fluorimetric protease assays use 7-amino-4-methlycoumarin (AMC or MUC) which has an amide group attached to one of the benzene rings instead of a hydroxide group, allowing for an amino acid to bond to the amide group via an amide bond (Table 1). Aminopeptidase enzymes hydrolyse the amide bond producing the amino acid and AMC. The latter, upon excitation by UV light at 330–380 nm, emits fluorescence at 440–480 nm that is read by a fluorometer. Due to their specificity, aminopeptidase assays do not provide an overall measurement of soil protease activity but rather a proxy to indicate rates of activity. Despite being expensive per gram of substrate, due to the low quantities needed (mg per assay) it is a cost-effective method (ca. £0.34 sample^{-1}; Table 1). Alkalinisation e.g. addition of NaOH is used in some protocols to increase the fluorescence of acidic solutions. However, German et al. (2011) found fluorescence was only difficult to quantify at pH values below 4.5. A geographical analysis of the use of fluorescent substrates revealed North America to have the highest proportion of fluorimetric to colorimetric analyses (see Fig. 2; Fig. S3).

3.1.2. Colorimetric-based protease assays

Casein is a milk-derived phosphoprotein substrate with a very high molecular weight, which can be broken down by a range of proteases (e.g. endo- and exoproteases) to produce peptide chains and amino acids (Table 1; Dewan et al., 1974; Landi et al., 2011). The Folin-Ciocalteu reagent is the most common chromophore reagent used to determine the quantity of breakdown products (used in a ca. 40% of colorimetric studies collected in this metadata) and reacts with tyrosine residues produced as a breakdown product by protease enzymes to form a blue chromophore. It was originally used as a protein assay by Lowry et al. (1951). As it reacts with tyrosine, the amount of tyrosine produced over a certain time can be measured and compared to a tyrosine standard. However, the Folin-Ciocalteu reagent also reacts with many other common compounds in soil and due to the complex nature of soil this means that several other compounds could be simultaneously measured (e.g. humic substances, buffers, chelating agents, and lipids) (Peterson, 1979).

BAA is a typical substrate for a trypsin-like enzyme producing NH_4^+ (Table 1) (Landi et al., 2011). NH_4^+ can then be measured colorimetrically using ninhydrin reagent (Ladd and Butler, 1972). Hydrindantin (reduced ninhydrin) is added directly to the reaction to prevent the precipitation of certain salts affecting accuracy. The carbonyl group on the ninhydrin reacts with nucleophilic groups on amino acids (e.g. NH_2-R) to form a ninhydrin chromophore of deep blue/purple colour (Moore and Stein, 1954). An amino acid standard e.g. leucine is used to determine the quantity of amino acids in the solution. However, as it also reacts with NH_4^+ which is immobilised by microorganisms in soil, toluene is often used to inhibit microbial activity (used in ca. 7% of colorimetric studies following the Watanabe and Hayano, 1995

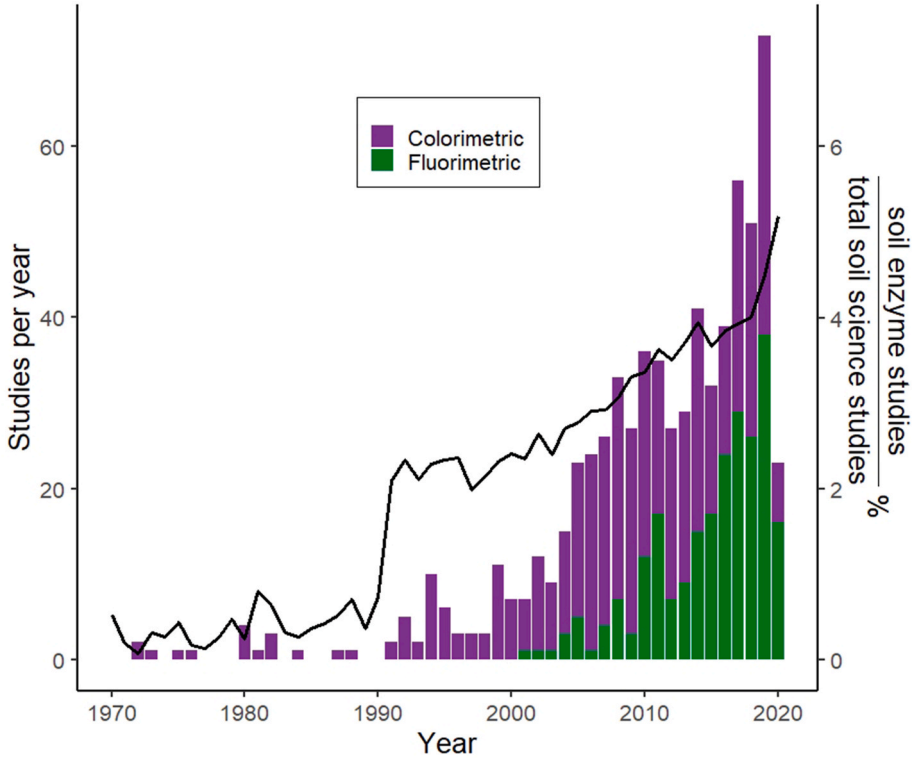

Fig. 1. Number of studies that have measured soil protease activity using colorimetric and fluorimetric techniques between 1970 and 2020 and that were considered within this systematic review. The black line represents the number of soil enzyme studies published each year as a percentage of total soil science studies published each year.

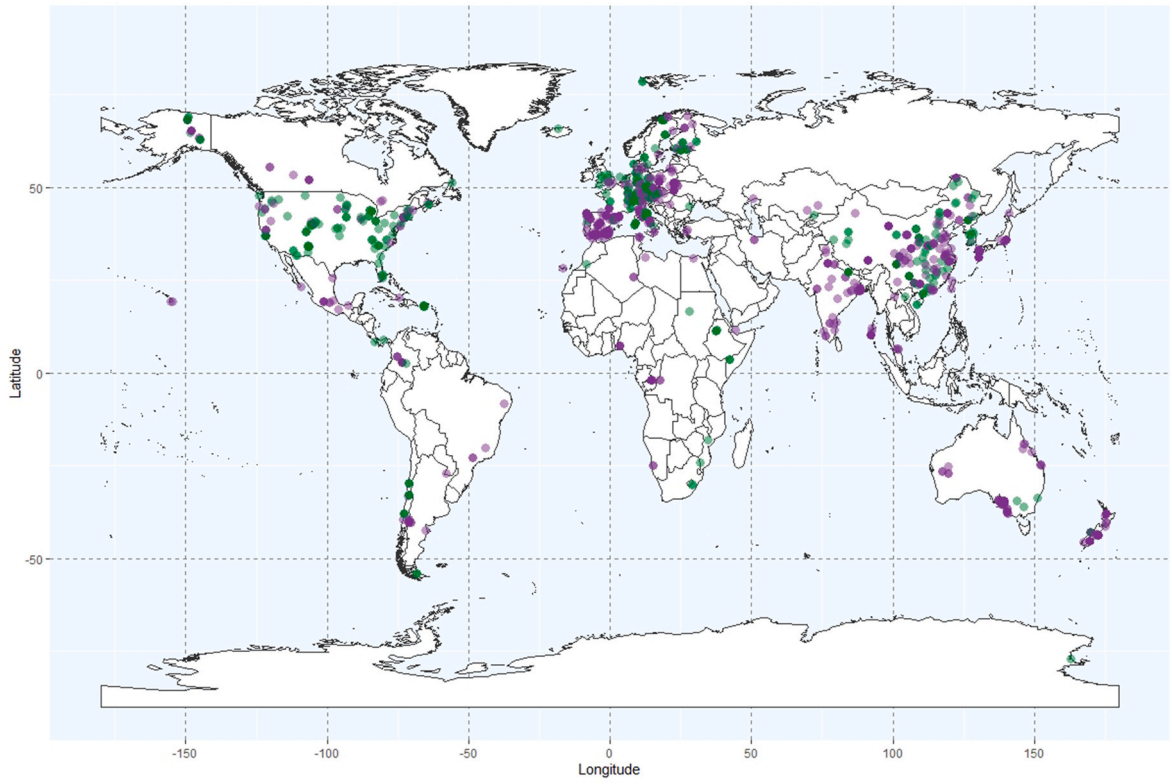

Fig. 2. Geographical location where soil protease activity has been measured either colorimetrically (*n of studies* = 393, purple symbols) or fluorometrically (*n of studies* = 179, green symbols). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Summary of substrates used to measure soil protease activity and their basic properties. Leucine-7-amino-4-methlycoumarin and glycine *p*-nitroaniline have been used as examples for AMC and pNA respectively as these are the most common forms for the substrate group (Table S5).

Substrate	Method	Protease measured	Hydrolysis mechanism	EC number	Product measured	Cost per sample (£)[a]	Main studies cited for protocol
Leucine-7-amino-4-methylcoumarin (AMC)	Fluorimetric	Leucine aminopeptidase	Exopeptidase (N-terminus)	3.4.11.	AMC	0.34	Bell et al. (2013) Saiya-Cork et al. (2002)(Marx et al., 2001)(Vepsäläinen et al., 2001)
Casein	Colorimetric	Trypsin	Endopeptidase	3.4.21–25	Tyrosine	0.002	Ladd and Butler (1972) Nannipieri et al. (1980) Guan (1986)
N-benzoyl L-arginine amide (BAA)	Colorimetric	Trypsin	Endopeptidase	3.4.21–25	NH_4^+	0.3	Nannipieri et al. (1980) Ladd and Butler (1972)
Glycine *p*-nitroaniline (pNA)	Colorimetric	Glycine aminopeptidase	Exopeptidase (N-terminus)	3.4.11.-	pNA	0.4	Sinsabaugh et al. (1993) Allison and Jastrow (2006)
Benzyloxycarbonyl phenylalanyl leucine (Z-phe-leu)	Colorimetric	Carboxypeptidase	Exopeptidase (C-terminus)	3.4.21–25	Leucine	0.13	Ladd and Butler (1972) Hayano (1993)

[a] Costs per sample was calculated according to the cost of the substrate (£) from Merck and the typical amount used per sample.

method). Reiskind et al. (2011) found toluene to only reduce immobilisation in organic tundra soils with no effect in the mineral tundra soils also tested. Thus, studies using ninhydrin reagent are likely to also be strongly impacted by microbial immobilisation of substrate released by protease action. No studies accounted for this in a quantitative way.

Z-phe-leu is a low molecular weight substrate also typically measured using the ninhydrin reagent (Ladd and Butler, 1972). It is hydrolysed by carboxypeptidases to produce leucine which is then measured colorimetrically. Due to using the same reagent as BAA (ninhydrin reagent), Z-phe-leu can also react with NH_4^+ and thus could be measuring microbial mineralisation as well as protease activity. Furthermore, the products of Z-phe-leu cleavage may also be consumed by the microbial biomass leading to an underestimation of production rate. Compared with the other substrates in this study, Z-phe-leu has the least amount of information on its mechanism and optimised conditions.

Lastly, p-nitroaniline (pNA) is a chromophore commonly used to measure aminopeptidase activity in soil (Sinsabaugh et al., 1993). The assay works similarly to AMC whereby the chromophore is bonded to an amino acid (e.g. glycine and leucine) and when this bond is hydrolysed by an aminopeptidase it turns purple (absorbance measured at ca. 410 nm) (Table 1). Like AMC assays, aminopeptidase assays do not provide an overall measurement of soil protease activity. This assay can be

carried out via bench- and microplate-scale making it versatile (Deng et al., 2013). Colorimetric-based assays have remained a popular method despite the rise in fluorimetric assay use, accounting for 60% of the total soil protease studies (Fig. 1).

3.2. Laboratory-optimised versus field-relevant protease assay conditions

3.2.1. Assay pH versus soil pH

The difference between assay pH and field soil pH (δpH) is shown in Fig. 3a. Except for BAA and Z-phe-leu, most assays were measured using an assay pH within 0–0.5 units of the actual soil pH (303 assays). For BAA and Z-phe-leu, the most common δpH was between 0.5 and 1 unit. For BAA and casein, ca. half of the assays measured a δpH greater than 1 and around a third of AMC and pNA and 16% of Z-phe-leu assays. A large proportion of studies ($n = 121$) did not report either assay pH or soil pH, meaning δpH could not be calculated. Generally, δpH was positive, meaning that the assay pH used was higher than the actual soil pH. The difference between assay pH and the pH optima (δpH) is shown in Fig. 3b. Except for BAA where two thirds of the assays were recorded at the pH optima (0–0.5 δpH), the majority of assays were measured at greater than 1 unit of pH from the optima (66–89%). Generally, δpH was positive for BAA, casein and Z-phe-leu meaning that the assay pH used was higher than the optimum pH measured and negative for AMC and pNA assays. This was probably due to the large number of AMC assays following Saiya-Cork et al. (2002) which used an assay pH of 5, whilst the majority of casein assays followed the method of Ladd and Butler (1972) which used an assay pH of 8.1.

3.2.2. Assay temperature versus soil temperature

The difference between assay temperature and MAT (δtemperature) is shown in Fig. 4. A small proportion of assays ($n = 71$) measured protease activity at a temperature close to their MAT (0–5 °C difference). Of these, no assays involved either BAA or Z-phe-leu. Generally, δtemperature was positive, meaning that the assay temperature used was higher than the actual MAT at the site where the sample was collected. Between 60 and 95% of assays for all substrates were measured at an assay temperature >10 °C higher than the soil's MAT. Up to 24% of assays did not report either assay temperature or MAT meaning δtemperature could not be calculated.

3.3. Numerical range of protease activity at a global scale

Mean soil protease activity ranged between 0 and 15 million nmol product g^{-1} h^{-1}, (Fig. 5). Whilst all four colorimetric substrates showed a density peak at ca. 1000 nmol product g^{-1} h^{-1}, the fluorimetric substrate AMC had a lower density peak at ca. 100 nmol product g-1 h^{-1}. Across all substrates, around 60% of the data lay between 0 and 1000 nmol product g^{-1} h^{-1} and 80% lay between 0 and 5000 nmol product g^{-1} h^{-1}. In addition, we observed large interstudy variations in soil protease activity with a mean standard error of ±74,000 nmol product g^{-1} h^{-1} and intra-study variation with a mean standard error of ±8,500 nmol product g^{-1} h^{-1} (Table 2).

3.4. Natural variation of protease activity at a global scale

Overall, there were few significant associations between environmental (MAT and soil pH) or methodological factors (assay temperature and pH) and protease activity and those that were significant ($p < 0.05$) had R^2 values < 0.32 (Fig. 6, Table 3). The magnitude of change of any environmental or methodological factor on protease activity was small, 0.02–0.52 (on a $\log_{10}$ scale) equating to 1.0–3.3 nmol product g^{-1} h^{-1} increase or decrease in protease activity (Table 3), compared to the large

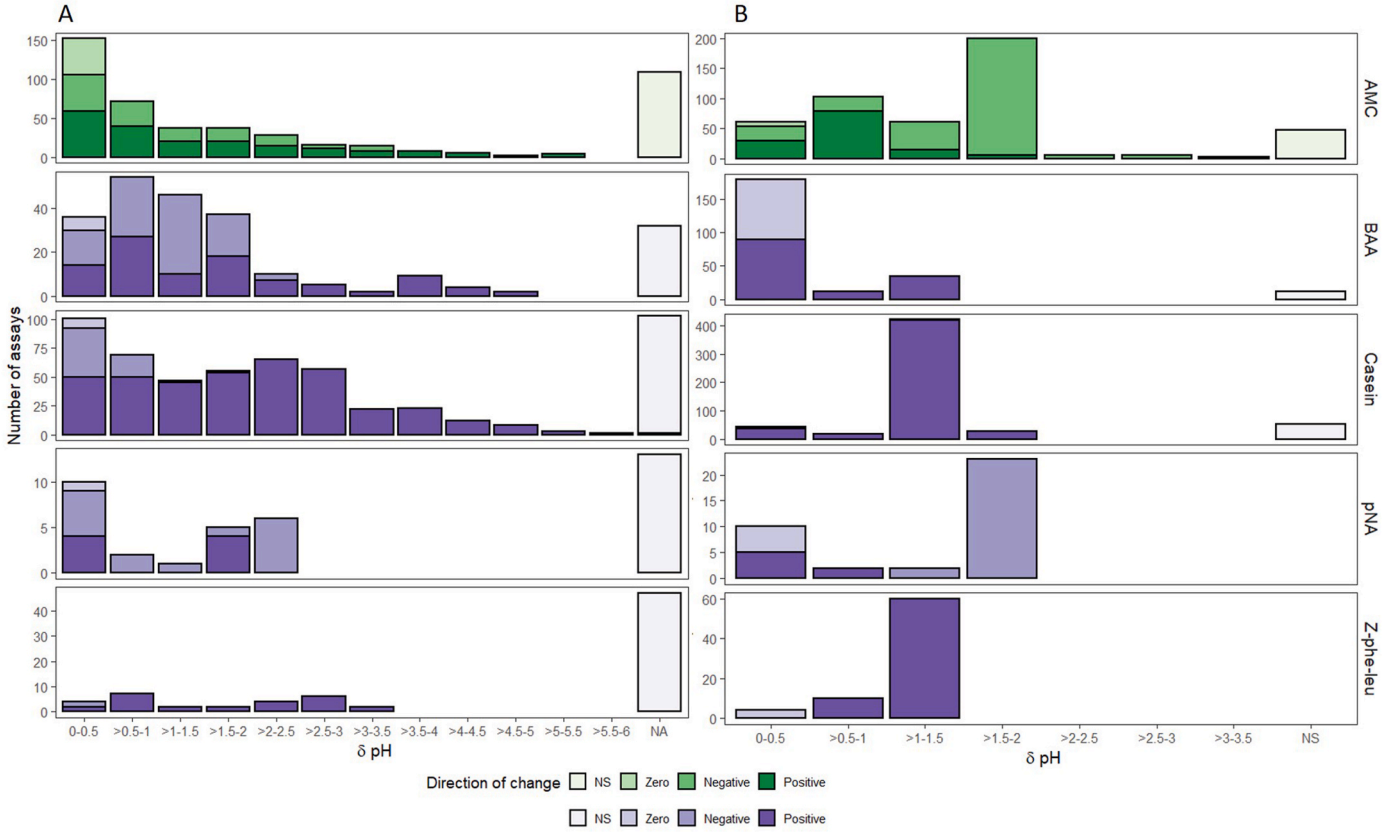

Fig. 3. Number of assays for each δpH for the A) difference between the assay pH and soil pH, and B) difference between the assay pH and pH optima of 7 (*n of studies* = 173, 99, 262, 21, 18 studies for AMC, BAA, casein, pNA and Z-phe-leu substrates, respectively). Colorimetric substrates are shown in purple and fluorimetric substrates are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

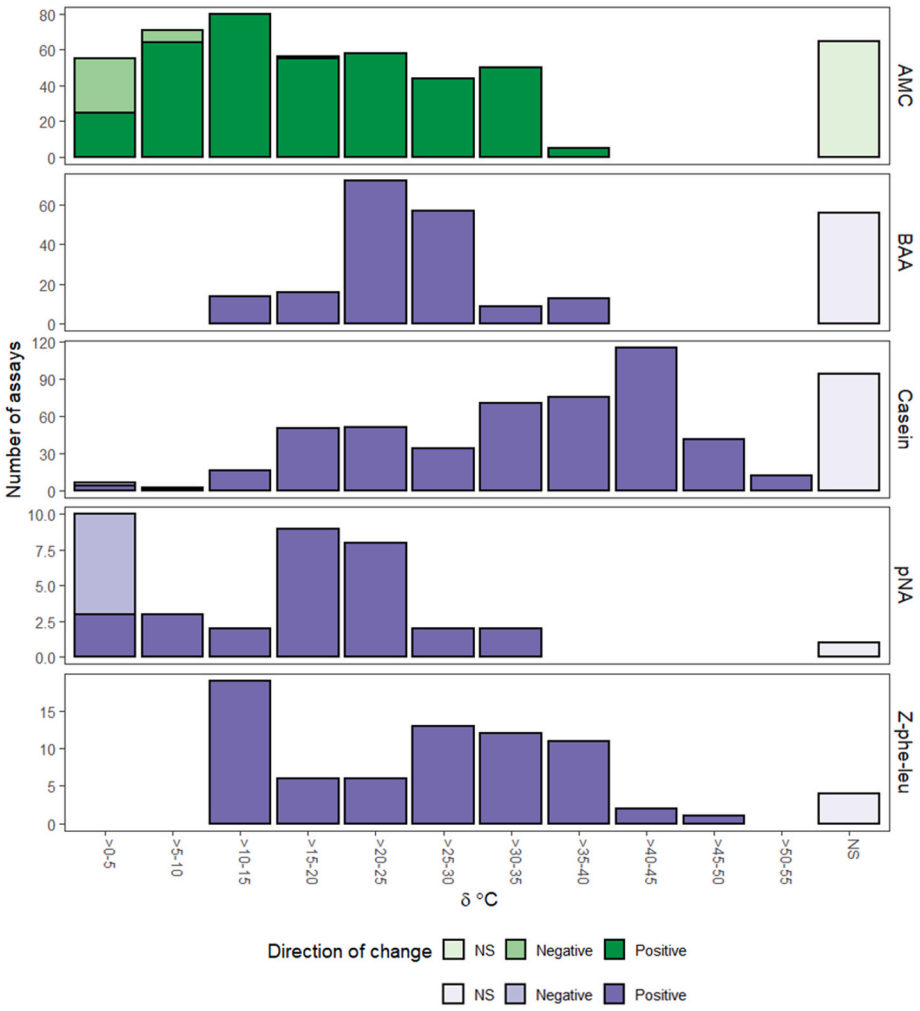

Fig. 4. Number of assays for each δtemperature (°C) between the assay temperature and mean annual temperature (MAT) (*n of studies* = 173, 99, 262, 22, 22, for AMC, BAA, casein, pNA and Z-phe-leu substrates, respectively). Colorimetric substrates are shown in purple and fluorimetric substrates are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

standard error observed between studies (Table 2).

4. Discussion

4.1. Colorimetric- versus fluorimetric-based protease assays

Only a few studies used more than one substrate simultaneously and so it was not possible to reliably determine correlations between protease activity measurements from different substrates. However, the small sample of data we obtained showed strong correlations between leucine-AMC, alanine-AMC and tyrosine-AMC but generally weaker correlations between colorimetric substrates (Fig. S4). All AMC substrates measure aminopeptidase activity and thus it is not surprising that the different substrates show strong agreement, while colorimetric substrates measure a wider range of different proteases. Typically, one protease-specific substrate is used as a proxy for total soil protease activity, yet all proteases will not have the same abundancy, activity, kinetic parameters, catalytic mechanism or ecological function (Vranová et al., 2013). This may explain why measurements of protease activity are generally higher with colorimetric substrates in comparison to fluorimetric substrates. With the measurement of soil protease activity becoming increasingly popular in soil enzyme studies (Fig. 1) it shows the importance of choosing the right protocol. In terms of ease, accuracy, reliability and increasing popularity, fluorimetric assays using AMC are considered the best choice (German et al., 2011; Nannipieri et al., 2017). In addition, the optimal pH and temperature conditions of the substrates that target multiple proteases and proteases with broad specificity (e.g. BAA and casein) are based on a limited number of older soil studies (e.g. Ladd, 1972; Ladd and Butler, 1972). In contrast, more recent work has been conducted on leucine aminopeptidase (e.g. Niemi and Vepsäläinen, 2005; Puissant et al., 2019). However, as this method only targets aminopeptidases it could be missing key soil biochemical pathways that involve other proteases. The use of microarrays to determine the activity of many types of proteases simultaneously would allow for a more holistic overview and quantitative assessment of protein turnover in soil (Sieber et al., 2004; Uttamchandani et al., 2005).

4.2. Laboratory-optimised versus field-relevant protease assay conditions

Numerous reviews have recommended that soil enzyme activity is best measured under field-relevant conditions (Burns et al., 2013; German et al., 2011; Nannipieri et al., 2017). Since the advice of German et al. (2011) was published, 30% of protease assays were neither conducted within 1 unit of pH of field conditions nor optimised for pH. However, Nannipieri et al. (2017) pointed out problems that occur when mimicking soil pH: 1) pH is heterogenous at the microscale (e.g. mineral surfaces) and macroscale (e.g. rhizosphere vs. bulk soil), and 2) pH is commonly measured in a soil/water suspension which can range markedly depending on season, land use and water source. Therefore, they suggest it would be best practice to measure soil protease activity at

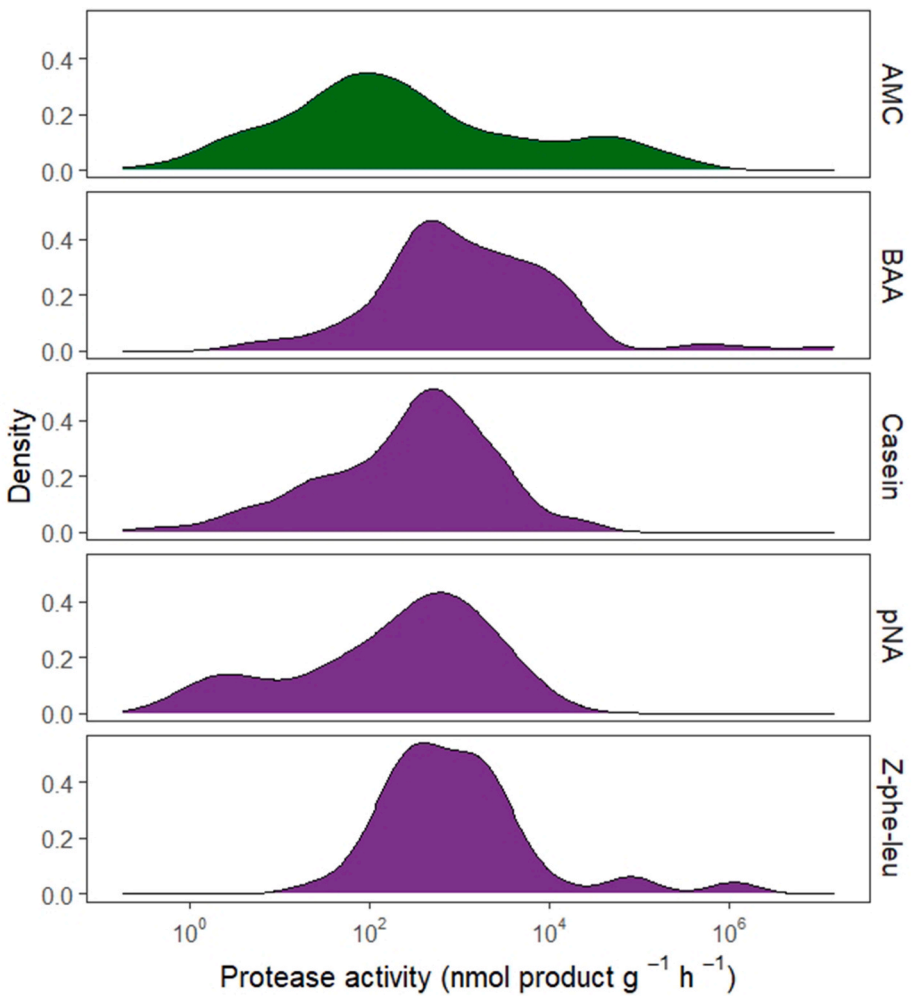

Fig. 5. Density distribution of the mean soil protease activity (nmol product g^{-1} h^{-1}) on a $\log_{10}$ scale for each substrate with outliers removed (*n of studies* = 162, 98, 244, 22 and 22 for AMC, BAA, casein, pNA and Z-phe-leu, respectively). Outliers were removed by estimating the maximum activity possible for the assay based on the amount of substrate added (i.e. theoretically impossible values where the reported protease activity exceeded the amount of substrate added were not deemed scientifically credible and were thus omitted). Colorimetric substrates are shown in purple and fluorimetric substrates are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Summary statistics of soil protease activity and associated environmental and methodological factors used in the linear regression model across 929 assays (n = 105, 79, 186, 14 and 11 studies for AMC, BAA, casein, pNA and Z-phe-leu, respectively).

Parameter	Mean	Min	Max	Median	Lower quartile	Upper quartile
Protease activity (nmol product g^{-1} h^{-1})	18,601	0.18	4,535,469	435	79	2,252
Interstudy standard deviation	74,496	0	35,880,000	56	9	351
Intra-study standard deviation	8,517	1.6	476,141	8,079	70	1,618
Soil pH	6.2	2.6	9.3	6.2	5.2	7.5
MAT (°C)	12	−14	30	13	7.5	16
Assay temperature (°C)	38	2	55	40	30	50
Assay pH	7.2	4.5	9	7.8	4.5	8.1

both field pH and optimised pH conditions. Unfortunately, to date, no published studies collected in our metadata have heeded this advice. Studies have observed microbial adaption to the edaphic environment, suggesting that optimised conditions may vary environmentally too (Allison et al., 2018; Puissant et al., 2015, 2019). Therefore, it is important to measure field soil protease activity under the environmental conditions when sampled in order to minimise variations that occur temporally. Best practice would be to determine V_{max} along a pH range in order to integrate enzyme activities into a model to evaluate the effect of environmental change of soil pH on soil protease activity. However, this is time consuming and thus the minimum practice is to 1) always report the pH of the assay and soil to allow for correction of the effect of using a different pH to the field condition, and 2) report the objective of the protease assay stating whether it aims to mimic field conditions or optimum enzyme conditions.

Temperature of the assay follows a similar trend to the assay pH with the majority of studies not using field-relevant conditions. There is no set value of optimised temperature for soil protease assay. Studies have shown that microorganisms are adapted to different temperatures as a result of their microclimate which will determine their temperature optima (German et al., 2011; Machmuller et al., 2016; Puissant et al., 2015). We used MAT as a proxy for soil temperature due to the lack of recording of field soil temperatures. However, MAT does not always represent the seasonal fluctuations in temperature which can affect protease activity (Koch et al., 2007; Puissant et al., 2015). Thus, when measuring soil protease activity under field conditions we suggest using soil temperature recorded as close to the assay as possible. Most of studies try to estimate protease activity to understand and quantify soil processes, therefore; measuring activity at the temperature as close as the field is essential. This is far from the case based on our observed

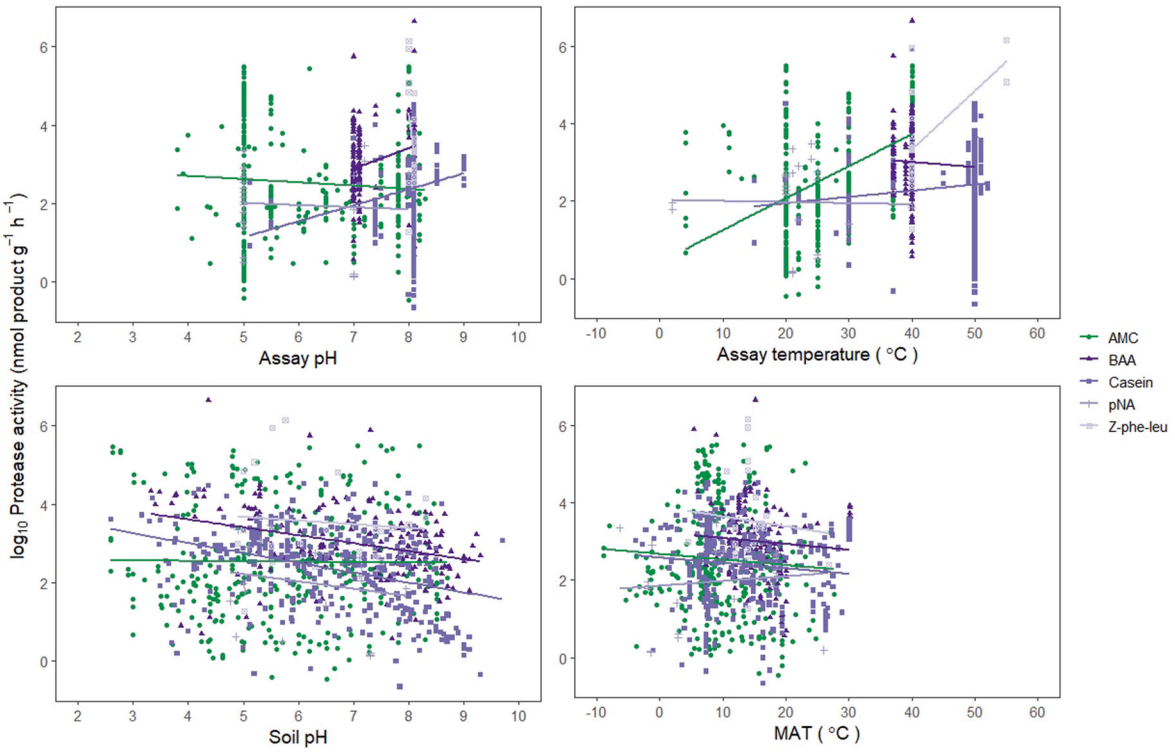

Fig. 6. Relationship between methodological (assay pH and assay temperature [°C]) or environmental (soil pH and mean annual temperature [MAT, °C]) factors and soil protease activity (log_{10} nmol product g^{-1} h^{-1}) for five different protease substrates (n = 105, 79, 186, 14 individual studies for AMC, BAA, casein, pNA and Z-phe-leu, substrates, respectively). Solid lines trace a linear regression fit (a summary of regression analyses can be found in Table 3). Green symbols indicate fluorimetric substrates and purple symbols indicate colorimetric substrates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Summary of linear regression model of the relationship between environmental (MAT and soil pH) or methodological (assay pH and temperature) factors and log_{10} protease activity for each substrate. Values in bold are significant at $p < 0.05$.

Factor	Substrate	Log_{10} protease activity		
		Line equation	R^2	p
MAT	AMC	$y = 2.6 - 0.01x$	0.0001	0.86
	BAA	$y = 3.2 - 0.02x$	0.006	0.29
	Casein	$y = 2.5 - 0.01x$	0.007	0.09
	pNA	$y = 1.9 + 0.01x$	0.01	0.63
	Z-phe-leu	$y = 3.9 - 0.03x$	0.007	0.70
Assay temperature	AMC	$y = 0.43 + 0.08x$	0.20	**<0.001**
	BAA	$y = 3.5 - 0.01x$	0.0004	0.78
	Casein	$y = 1.6 + 0.02x$	0.01	0.03
	pNA	$y = 2.0 - 0.003x$	0.0004	0.92
	Z-phe-leu	$y = -2.7 + 0.15x$	0.32	**0.005**
Soil pH	AMC	$y = 2.6 - 0.01x$	0.0001	0.86
	BAA	$y = 4.4 - 0.20x$	0.12	**<0.001**
	Casein	$y = 4.0 - 0.25x$	0.14	**<0.001**
	pNA	$y = 3.2 - 0.19x$	0.04	0.38
	Z-phe-leu	$y = 4.1 - 0.09x$	0.01	0.67
Assay pH	AMC	$y = 3.0 - 0.08x$	0.01	0.21
	BAA	$y = -0.82 + 0.52x$	0.05	**0.002**
	Casein	$y = -0.90 + 0.41x$	0.03	**<0.001**
	pNA	$y = 2.3 - 0.05x$	0.003	0.08
	Z-phe-leu	$y = 87 - 10x$	0.16	0.06

results that 10% of assays were incubated at an assay temperature within 10 °C of the MAT. Most assays are measured at higher temperatures than the field in order to increase the rate of reaction to be closer to optimum conditions. However, this leads to an inflated measurement far from what is happening *in situ*. This can lead to false or misleading conclusions being drawn, especially if the activity of isoenzymes adapted to cold versus hot temperature are compared at the same laboratory assay temperature (Wallenstein et al., 2009). Crude estimations of field-temperature protease activity, calculated from mean soil protease activity using MAT reported in the studies and assuming a Q_{10} of 2 (von Lützow and Kögel-Knabner, 2009), suggest that protease activity measured at field temperature would be more than 5 times lower (Supplementary information 1). Some studies have determined the optimum temperature of the protease they are measuring at around 40–60 °C (Ladd and Butler, 1972; Nannipieri et al., 1982). Ideally, the temperature response of protease activity should be measured along a temperature gradient to determine the temperature sensitivity of the enzyme which is driven by many environmental variables e.g. interaction with the soil structure and ecological niche of the microbial pool that produce isoenzymes (Wallenstein et al., 2010). Again, this is time consuming and so, when measuring protease activity in relation to soil processes, activity should be measured at temperatures which best reflect field conditions at the time of sampling.

In the two years following the last major review into soil enzyme activity methods by Nannipieri et al. (2017), one-third of studies did not report one or more of: assay temperature, assay pH and soil pH and only a handful reporteded soil temperature. This is despite Nannipieri et al.'s (2017) reiteration of the work of Dick (2011) stating that these factors are key for establishing accurate and standardised methods in soil enzymology. Based on a quantitative assessment of different methodological approaches we reiterate points made in previous reviews of enzyme activity methods; studies must be transparent in the reporting

key factors that will influence the accuracy and interpretation of soil enzyme activity. From the studies obtained in this synthesis, it is possible that as the number of studies measuring protease activity and soil enzymes has increased each year, soil protease activity is more often being used as a soil quality indicator and a basic soil property amongst a range of key enzyme activities involved in soil C-N-P cycling (e.g. phosphatases and β-glucosidase; Boafo et al., 2020). Therefore, as the focus of these studies was not of soil protease activity, it might be that time has not been taken to research and develop the most appropriate protocol. It is fundamental to report soil and assay parameters of temperature and pH that are known to influence protease activity. Furthermore, research on how pH and temperature regulate soil protease activity by measuring response curves will aid the determination of potential field-relevant protease activity and estimates of ecological flux and feed biogeochemical models. This will reduce the variation in soil protease activity measurements due to methodological bias and help us better understand ecological drivers.

The choice between field-relevant or laboratory-optimised conditions depends on the aim of the study. For example, a focus on proteases and their kinetic and thermodynamic properties would warrant an optimised approach whilst studies measuring proteases from an ecological perspective should opt for field conditions (Burns et al., 2013; Nannipieri et al., 2017). Although, the adoption of field conditions represents the soil pH and temperature more accurately, it is still not a measure of *in situ* protease activity due to the use of substrates at high concentrations that saturate the system and fail to account for factors that influence protein availability to microbes in soil (e.g. sorption to the solid phase, interaction with humic substances, diffusion, pore tortuosity). Determining Michaelis Menten kinetic parameters, V_{max} and K_m (Miller et al., 2001), and their sensitivity to environmental factors (e.g. pH, temperature) allows us to take into account the effect of substrate concentration on enzyme reaction velocity and would help to better model *in situ* activity. In addition, more studies that isolate, purify, and characterise enzymes in soils will help measure the enzyme kinetic parameters and how the environment affects these. Combined with a genetic approach to determine protease gene expression in soil, recombinant proteins could be produced, and their kinetic parameters measured. The main point here is to explicitly state which approach was used in a study. However, regardless of the approaches used justification must be made, which was rare for the studies reviewed here.

4.3. Natural variation of protease activity at a global scale

We observed a large variation in global protease activity of 0–15 million nmol product g^{-1} h^{-1} even after the exclusion of invalid results. Together with the lack of standardisation and reporting of associated abiotic and biotic data, it suggests that many values could be unreliable. Also, there is an unknown variable of calculation error of protease activity which cannot be determined due to inaccessible raw data and lack of reporting of equations used. As around 80% of the data observed in this synthesis lay between 0 and 5000 nmol product g^{-1} h^{-1} we recommend this range as initial guidance for future studies to assess the validity of their results. Based on a review by Kallenbach and Grandy (2011), soil microbial biomass-C ranges between 43 and 2125 mg C kg^{-1} with an average of 365 mg C kg^{-1} in agricultural soils. As microorganisms exude proteases into soil, it would be expected that the number of proteases would limit activity to a smaller range than the 0–15 million nmol product g^{-1} h^{-1} observed in this synthesis (Noll et al., 2019). However, microbial biomass-C estimates are composed of both active and dead microorganisms. In hotspots, microbial activity can be 2–10 times more than in the bulk soil (Kuzyakov and Blagodatskaya, 2015). This could result in values over the 5000 nmol product g^{-1} h^{-1} threshold value we suggest. However, it is unlikely that hotspot activities would be 900 times higher than the threshold suggesting an error in the methodological protocol. The reliability and accuracy of results cannot be determined until a standardised protocol is established and followed. We recommend using a control of pure protease to ensure activity is within the range of what should be expected and referring to German et al. (2011) for a step-by-step guide to determining activity and unit.

There was a difference in soil protease activity density peaks between fluorimetric and colorimetric analyses (100 nmol product g^{-1} h^{-1} compared to 1000 nmol product g^{-1} h^{-1}). This could be due to the methodologies chosen for colorimetric analysis which tend to use higher assay pH and temperature conditions (Table S6). This is also apparent from Fig. 6 where a bias in assay pH and temperature conditions can be seen between fluorimetric and colorimetric analysis. AMC and pNA have the similar assay conditions (~pH 5.9 and 23.5 °C) which may explain the similar distribution in Fig. 6 compared to other colorimetric substrates and AMC. Despite the similar assay pH and temperature conditions between AMC and pNA, the peak of the density distribution for soil protease activity was 100 times more for pNA. A study comparing equivalent substrates to AMC and pNA, MUF and pNP, used for β-d-glucosidase, N-acetyl-β-d-glucosaminidase, and acid phosphomonoesterase assays, found pNP assays to result in 107–412% more activity (Deng et al., 2013). This is because of the lower sensitivity of pNP compared to MUF microplate assays. More work is needed to determine the underlying reasons for the disparity between substrates specifically for protease activity.

4.4. Potential factors to explain global variation in soil protease activity

The variation of protease activity was not accounted for by environmental or methodological factors analysed in this study and, thus, we were not able to fully address our aim of identifying potential factors that may help explain the natural variation in protease activity. Our study focused on pH and temperature as key environmental variables; however, other studies have found relationships between soil organic carbon, clay content, soil microbial biomass and soil moisture with enzyme activities (e.g. Bonmati et al., 1991; Geisseler et al., 2010). Several studies have also reported seasonal effects on enzyme activities due to temperature and precipitation changes which were not possible to investigate in this study (Brzostek and Finzi, 2012; Wallenstein et al., 2009). Data for the parameters such as soil organic carbon and clay content are often not reported together with protease activity. This causes difficulties in inferring relationships between the parameters. In addition, it has been shown that protein mineralisation and protease activity are influenced by many interacting factors and, thus, a suit of factors should be reported (Noll et al., 2019; Greenfield et al., 2020b). These additional parameters measured, on top of pH and temperature, should be determined according to the research aims of the study. Studies measuring proteases from an ecological perspective should opt for field conditions e.g. for protease activity in relation to soil C and N cycling it is important to measure microbial biomass, organic and inorganic N content, C:N ratio, texture and moisture content (Noll et al., 2019; Greenfield et al., 2020b). A focus on proteases and their kinetic and thermodynamic properties would warrant an optimised approach measuring protease activity along pH and temperature gradients to determine optima.

In the case of methodological artefacts, the variation in protocol design and conditions is likely to cause high variation in global protease activity data. The large inter-study error suggests that the observed variation originates from the methodology. This synthesis has not investigated the effect of substrate concentration and pH buffer used (both type and molarity). Substrate concentration ranged from 0.01 to 20 mM in the metadata collected in this study (Greenfield et al., 2021), however, without insight into the kinetic parameters we cannot gauge whether this would lead to substrate saturation for each specific soil. Buffer choice is likely to affect the solubilisation of enzymes within the soil and thus their activity (Greenfield et al., 2018; Fairbridge et al., 2008). It may also affect the solubilisation of protease inhibiting substances (e.g. humic substances). Protease assays carried out below the substrate saturation could lead to underestimation of protease activity

(German et al., 2011). A wide range of buffers are used with 15 different buffers and 16 different molarities (1–1000 mM) were used in assays collected in our metadata (Greenfield et al., 2021). Without standardisation and reporting of associated abiotic and biotic data it is difficult to determine the underlying drivers of global protease activity (Burns et al., 2013; Nannipieri et al., 2017). Therefore, we stress the importance of developing an international standardised method, explicitly stating soil and assay properties, and carefully choosing field or optimised conditions. Once these recommendations are adopted it will be easier to ascertain the potential factors that influence variation in soil protease activity.

5. Conclusions

Many studies assessing enzyme activity protocols have concluded that standardised methods must be used (Burns et al., 2013; Deng et al., 2013; Dick et al., 2018; Nannipieri et al., 2017). However, we present evidence that a wide range of modified (non-standardised) methods continue to be used in most studies. When compared to average microbial biomass-C in soil, it is likely that a significant number of studies present protease results that are not valid. Thus, we hope our quantitative evidence, showing the range of methods used to date and the variation and error this has caused in measurements, encourages the soil enzyme research community to adopt the standardised practice we recommend. Also, we stress the importance of fully disclosing the assay protocol conducted with all the conditions stated in the methods. When measuring soil protease activity, we recommend the following:

1. Transparent reporting of assay conditions and soil characteristics, particularly pH and temperature as well as soil moisture, microbial biomass, texture and organic carbon content.
2. As a minimum, conduct the assay at either field or optimised pH and temperature based on the aims of the study.
3. Check that measurements lie between 0 and 5000 nmol product g^{-1} h^{-1} to prevent reporting of erroneous values.

Abiotic and biotic factors affect parameters like enzyme kinetics and temperature sensitivities which feed global biogeochemical models. Therefore, precise reporting of abiotic and biotic associated data will help increase understanding the ecological drivers of protease activity and refine parameterisation of global biogeochemical models with new data on enzymatic mechanisms and kinetics. Future methodological developments should focus on creating microarrays that can assay multiple types of proteases simultaneously under the same pH and temperature. This will allow standardisation of protease activity measurements. Furthermore, microarrays reduce the need to use different substrates that use different protocols and conditions that make comparing protease activity unreliable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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